CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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MM09-A2

Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline—Second Edition

This document addresses diagnostic sequencing using both automated capillary-based sequencers and massively parallel sequencing instruments. Topics include specimen collection and handling; isolation and extraction of nucleic acid; template preparation; sequence generation, alignment, and assembly; validation and verification; ongoing quality assurance; and reporting results.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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For further information on committee participation or to submit comments, contact CLSI.

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Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline—Second Edition

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Abstract

Sequencing methods for genotyping have moved from the research laboratory into the clinical laboratory. Sequencing is an assay format of choice for very high-complexity genotyping, especially when hundreds or thousands of bases of genetic sequence are analyzed. Clinical and Laboratory Standards Institute document MM09-A2—*Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline—Second Edition* addresses diagnostic sequencing using both automated capillary electrophoresis sequencers and massively parallel sequencing instruments. Topics covered include specimen collection and handling; isolation and extraction of nucleic acid; template preparation; sequence generation, alignment, and assembly; validation and verification; ongoing QA; and reporting results.

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Foreword

Significant advances in clinical diagnostic sequencing prompted the development of the second edition of this document. The original guideline focused primarily on the establishment and use of Sanger sequencing in the clinical laboratory, which at the time was the principal means for the collection of DNA sequence data. Since publication of the first edition, massively parallel sequencing (MPS) has become part of the clinical laboratory repertoire. MPS is a catchall term that includes a number of technologies that can generate a large amount of digital sequences. A feature that distinguishes MPS from Sanger sequencing is the heavy reliance on informatics to process the raw data derived from the instrument into interpretable DNA sequence. Laboratories have already begun to establish testing using MPS for heritable conditions, cancer, and infectious diseases. Advances in other areas suggest that applications for analysis of genome-wide methylation patterns, microbiomes, metagenomics, and transcriptome sequencing are forthcoming.

This revised guideline provides additional details that address the implementation of MPS into the clinical laboratory. In Section 5, users are initially oriented to the many sequencing technologies and currently available applications; the contents of this section represent the technologies available at the time of this publication, and users are encouraged to seek out recent reviews for the latest updates in this rapidly evolving field. Information on the implementation of sequencing in the clinical laboratory has been significantly expanded. Other sections have been added that discuss issues relevant to setup, running, and QC of the instrumentation and considerations for informatics analysis. Test validation is discussed in greater detail, and a separate section on QA and QC was also created. The final section that addresses the reporting of results was revised primarily as a consequence of new guidance and resources that have become available since publication of the previous edition of MM09. Sections that address Sanger sequencing remain but have been updated as needed to reflect advances in practice. Sections addressing specimen collection and preparation for analysis were consolidated into a much shorter section (see Section 6) because these have become common laboratory practice and are covered in far greater detail in other referenced CLSI documents. This revision is designed to provide guidance to experienced and knowledgeable laboratory professionals to assist with the implementation of high-quality diagnostic sequence analysis in the clinical laboratory.

Key Words

Capillary electrophoresis, clinical sequencing, dideoxy-terminators, electrophoresis, gel electrophoresis, massively parallel sequencing, next generation sequencing, nucleic acid, polymerase chain reaction, Sanger sequencing

Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline—Second Edition

1 Scope

The intended users of this guideline are clinical laboratories involved in the development, validation, verification, and implementation of sequencing-based assays.

This guideline specifies recommendations for the sequencing process, including specimen collection and handling, isolation of nucleic acid, amplification and sequencing of nucleic acids, and general interpretation and reporting of genotyping results. It is the intent of this document to provide instruction for verifying that the sequence obtained is accurate and suitable for subsequent interpretation; to address general interpretation of the sequence; and to provide QA/QC considerations for each step of the process, as appropriate. It is also intended to assist laboratories in generating appropriate and efficient validation across sequencing methods and applications. Sanger-based DNA sequencing and general aspects of massively parallel sequencing (MPS) are addressed in this guideline with specific examples.

This guideline:

- Does not comprehensively address platform-specific issues, because sequencing technology is rapidly evolving
- Provides general guidance for interpreting sequencing results and does not address the medical interpretation for a given patient, which is under the purview of the health care provider
- Is relevant to germline, somatic, and microbiological applications in clinical settings

2 Introduction

Sequencing is an increasingly important tool for genotyping in molecular diagnostics. Sequencing is routinely used in genotyping infectious disease organisms such as HIV and hepatitis C virus (HCV). When typing tissue for transplantation, human leukocyte antigen (HLA) typing is also performed by sequencing. There are also a variety of applications of sequencing for oncology and for diagnosing heritable conditions. The widespread use of laboratory-developed, sequencing-based genotyping assays and commercially available sequencing-based genotyping kits spurred the development of the original guideline for the development, verification, validation, and implementation of sequencing-based assays.

The previous edition of this guideline focused on sequence analysis using dideoxy chain-terminating chemistry and capillary electrophoresis (CE) instrumentation. In recent years, a number of new technologies have been introduced commercially. One change that has occurred since the previous edition of the guideline was published is that some instruments and assays have received regulatory approval or clearance for Sanger or MPS clinical testing. For clinical tests that use these products, the process of test validation and establishing QC parameters is simplified relative to a corresponding laboratory-developed test. Additionally, there are an increasing number of clinical applications, especially for multigene panels applied to genetic disease, for specific applications in which minor population variants can be clinically important (eg, HIV tropism), and in oncology, where both broad coverage and low-level variant detection can be of value. Emerging fields, such as epigenetics and the study of the microbiomes, suggest that applications to additional complex clinical questions will only increase as sequencers are able to generate more information at lower cost. This infusion of new technology requires a fresh look at some of the subject matter covered for validation of CE sequencing applications, and also introduces new challenges in the areas of platform validation and appropriate data analysis and management. This document was

revised during a time of flux, with several technologies currently undergoing rapid development in both instrumentation and software.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. The Centers for Disease Control and Prevention address this topic in published guidelines that focus on the daily operations of diagnostic medicine in human and animal medicine while encouraging a culture of safety in the laboratory.[1] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.[2]

4 Terminology

4.1 A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, International Organization for Standardization (ISO), and European Committee for Standardization (CEN) documents; and that legally required use of terms, regional usage, and different consensus timelines are all important considerations in the harmonization process. In light of this, CLSI's consensus process for development and revision of standards and guidelines focuses on harmonization of terms to facilitate the global application of standards and guidelines.

In order to align the usage of terminology in this document with that of ISO and CLSI document QMS01[3] the terms *preexamination, examination,* and *postexamination* have replaced preanalytical, analytical, and postanalytical, respectively, when referring to the testing phases within the path of workflow in a laboratory.

4.2 Definitions

accuracy (of measurement) – closeness of agreement between a measured quantity value and a true quantity value of a measurand (JCGM 200:2012)[4]; **NOTE:** For nucleic acid sequence analysis, the overall accuracy refers to the closeness of the derived assembled sequence to the true sequence.

adapter (oligonucleotide adapter) – a short sequence of deoxynucleotides used to couple segments of oligonucleotide.

alignment – the process of lining up two or more sequences for the purpose of assessing the percent identity shared between sequences and/or constructing a contiguous sequence from overlapping smaller segments.

all base accuracy – calculated by determining the percentage of the bases called that agree with the expected base call in the reference sequence.

The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The quality management system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any health care service's path of workflow (ie, operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The QSEs are as follows:

Organization	Personnel	Process Management	Nonconforming Event Management
Customer Focus	Purchasing and Inventory	Documents and Records	Assessments
Facilities and Safety	Equipment	Information Management	Continual Improvement

MM09-A2 addresses the QSE indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section, beginning on page 122.

Organization	Customer Focus	Facilities and Safety	Personnel	Purchasing and Inventory	Equipment	Process Management	Documents and Records	Information Management	Nonconforming Event Management	Assessments	Continual Improvement
		M29				X EP07 EP15 GP27 GP29 MM01 MM03 MM05 MM06 MM13 MM14 MM17 MM18				GP27 GP29 MM05	GP27
MM19 MM20 QMS01	MM19 MM20 QMS01	MM19 MM20 QMS01	MM19 MM20 QMS01	MM19 QMS01	MM19 QMS01	MM19 MM20 QMS01	MM19 MM20 QMS01 QMS02	MM19 MM20 QMS01	MM19 MM20 QMS01 QMS11	MM19 MM20 QMS01	MM19 MM20 QMS01 QMS06 QMS12

Path of Workflow

A path of workflow is the description of the necessary processes to deliver the particular product or service that the organization or entity provides. A laboratory path of workflow consists of the sequential processes: preexamination, examination, and postexamination and their respective sequential subprocesses. All laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

MM09-A2 addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI Reference Materials section on the following page.

Preexamination				**Examination**			**Postexamination**	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
X	X	X	X	X	X	X	X	X
MM01	MM01	MM01	MM01	MM01	MM01	MM01	MM01	MM01
	MM03	MM03	MM03	MM03	MM03		MM03	
MM05			MM05	MM05	MM05	MM05	MM05	MM05
MM06	MM06	MM06	MM06	MM06	MM06	MM06	MM06	
	MM13	MM13	MM13					MM13
	MM19	MM19	MM19	MM19	MM19	MM19		
MM20	MM20	MM20	MM20	MM20	MM20	MM20	MM20	MM20
QMS01	QMS01	QMS01	QMS01	QMS01	QMS01	QMS01	QMS01	QMS01

Related CLSI Reference Materials*

EP07-A2 **Interference Testing in Clinical Chemistry; Approved Guideline—Second Edition (2005).** This document provides background information, guidance, and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.

EP15-A2 **User Verification of Performance for Precision and Trueness; Approved Guideline—Second Edition (2006).** This document describes the demonstration of method precision and trueness for clinical laboratory quantitative methods utilizing a protocol designed to be completed within five working days or less.

GP27-A2 **Using Proficiency Testing to Improve the Clinical Laboratory; Approved Guideline—Second Edition (2007).** This guideline provides assistance to laboratories in using proficiency testing as a quality improvement tool.

GP29-A2 **Assessment of Laboratory Tests When Proficiency Testing Is Not Available; Approved Guideline—Second Edition (2008).** This document offers methods to assess test performance when proficiency testing (PT) is not available; these methods include examples with statistical analyses. This document is intended for use by laboratory managers and testing personnel in traditional clinical laboratories as well as in point-of-care and bedside testing environments.

M29-A3 **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

MM01-A3 **Molecular Methods for Clinical Genetics and Oncology Testing; Approved Guideline—Third Edition (2012).** This document provides guidance for the use of molecular biological techniques for detection of mutations associated with inherited medical disorders, somatic or acquired diseases with genetic associations, and pharmacogenetic response.

MM03-A2 **Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition (2006).** This guideline addresses topics relating to clinical applications, amplified and nonamplified nucleic acid methods, selection and qualification of nucleic acid sequences, establishment and evaluation of test performance characteristics, inhibitors, and interfering substances, controlling false-positive reactions, reporting and interpretation of results, quality assurance, regulatory issues, and recommendations for manufacturers and clinical laboratories.

MM05-A2 **Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline—Second Edition (2012).** This guideline addresses the performance and application of assays for gene rearrangement and translocations by both polymerase chain reaction (PCR) and reverse-transcriptase PCR techniques, and includes information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

MM06-A2 **Quantitative Molecular Methods for Infectious Diseases; Approved Guideline—Second Edition (2010).** This document provides guidance for the development and use of quantitative molecular methods, such as nucleic acid probes and nucleic acid amplification techniques of the target sequences specific to particular microorganisms. It also presents recommendations for quality assurance, proficiency testing, and interpretation of results.

MM13-A **Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline (2005).** This document provides guidance related to proper and safe biological specimen collection and nucleic acid isolation and purification. These topics include methods of collection, recommended storage and transport conditions, and available nucleic acid purification technologies for each specimen/nucleic acid type. A CLSI-IFCC joint project.

* CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

Related CLSI Reference Materials (Continued)

MM14-A2 **Design of Molecular Proficiency Testing/External Quality Assessment; Approved Guideline—Second Edition (2013).** This document provides guidelines for a quality proficiency testing/external quality assessment program, including reliable databases; design control in the choice of materials and measurands; good manufacturing processes; documentation procedures; complaint handling; corrective and preventive action plans; and responsive timing of reports. A CLSI-IFCC joint project.

MM17-A **Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline (2008).** This guideline provides recommendations for analytic verification and validation of multiplex assays, as well as a review of different types of biologic and synthetic reference materials.

MM18-A **Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline (2008).** Sequencing DNA targets of cultured isolates provides a quantitative metric within which to perceive microbial diversity, and can serve as the basis to identify microorganisms. This document is an effort to catalyze the entry of molecular microbiology into clinical usage by establishing interpretive criteria for microorganism identification.
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MM19-A **Establishing Molecular Testing in Clinical Laboratory Environments; Approved Guideline (2011).** This guideline provides comprehensive guidance for planning and implementation of molecular diagnostic testing, including strategic planning, regulatory requirements, implementation, quality management, and special considerations for the subspecialties of molecular genetics, infectious diseases, oncology, and pharmacogenetics.

MM20-A **Quality Management for Molecular Genetic Testing; Approved Guideline (2012).** This document provides guidance for implementing international quality management system standards in laboratories that perform human molecular genetic testing for inherited or acquired conditions.

QMS01-A4 **Quality Management System: A Model for Laboratory Services; Approved Guideline—Fourth Edition (2011).** This document provides a model for medical laboratories that will assist with implementation and maintenance of an effective quality management system.

QMS02-A6 **Quality Management System: Development and Management of Laboratory Documents; Approved Guideline—Sixth Edition (2013).** This document provides guidance on the processes needed for document management, including creating, controlling, changing, and retiring a laboratory's policy, process, procedure, and form documents in both paper and electronic environments.

QMS06-A3 **Quality Management System: Continual Improvement; Approved Guideline—Third Edition (2011).** This guideline considers continual improvement as an ongoing, systematic effort that is an essential component of a quality management system. A continual improvement program may consist of fundamental processes and common supporting elements described in this guideline.

QMS11-A **Management of Nonconforming Laboratory Events; Approved Guideline (2007).** This guideline provides an outline and the content for developing a program to manage a health care service's nonconforming events that is based on the principles of quality management and patient safety.

QMS12-A **Development and Use of Quality Indicators for Process Improvement and Monitoring of Laboratory Quality; Approved Guideline (2010).** This document provides guidance on development of quality indicators and their use in the medical laboratory.

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